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Rapid report

The effect of excited state population in Photosystem II on the photoinhibition-induced changes in chlorophyll fluorescence parameters

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Abstract

The photoinhibition-induced changes in Photosystem II fluorescence parameters of spinach thylakoids were only slightly sensitive to the excited state population in Photosystem II antenna, as modulated by either quinone quenching or energy spillover. The possibility that this may be due to a small fraction of chlorophyll molecules which are poorly coupled to the antenna is discussed. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Photosystem II; Photoinhibition; Quinone quenching; Energy spillover; Antenna system

Plants in natural environments are often exposed to high photon flux densities which for a leaf can lead to absorption fluxes up to the order of one photon per photosystem per millisecond. As the intrinsic rate constants for the processes involved in plastoquinone oxidation are about one order of magnitude slower [1], this means that the Photosystem II (PS II) quinone acceptors are substantially reduced in high light conditions with a consequent low efficiency for primary photochemistry. Thus, under these conditions, light absorption rate is greater than the capacity to perform photosynthesis and photo-oxidative damage to PS II may ensue. This process, known as photoinhibition, has been greatly studied in recent years, and a number of mechanisms which are thought to protect PS II from photoinhibition have been proposed (for recent reviews see [2,3]). It is generally considered that an extremely important regulatory mechanism of this kind is based on the light induced development of quenching states within PS II, known as non-photochemical quenching [4–7]. These states, most of which are thought to be associated with the external light harvesting antenna complexes [7–10], are considered to protect PS II against photoinhibitory damage by lowering the excited state levels. This hypothesis is therefore based on the assumption that photoinhibition is strongly correlated with the excited state population in PS II antenna. In the present paper we critically examine this assumption by experimentally modulating the excited state population in PS II of isolated spinach thylakoids and examining the effect of this on the photoinhibitory alteration of PS II fluorescence parameters. It is observed that a significant part of the laboratory-induced photoinhibitory damage is not correlated with excited state levels.

Stacked thylakoids were prepared from freshly harvested spinach leaves, which had been dark

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adapted for 2 h, by homogenisation in a Tricine buffer (30 mM, pH 8.0) containing sucrose (0.4 M), NaCl (10 mM) and $MgCl_2$ (5 mM), as previously described [11]. Unless otherwise stated, photoinhibition was performed by exposing thylakoids, incubated in a 1-cm pathwidth spectrophotometer cuvette, to a brief (3 min) treatment with intense white light (0.33 W/cm^2) from a xenon lamp (Applied Photophysics), at 4°C. Under these conditions, i.e., without addition of ascorbate, reversible, high-energy quenching is small and does not influence the present analysis. Ultraviolet and infrared wavelengths were removed by use of a Calflex heat filter plus a 3-cm optical pathlength water filter. Thylakoids were usually treated at a chlorophyll concentration of 4 μg/ml although on some occasions a much higher concentration (100 μg/ml) was used. All fluorescence measurements were performed at 4 μg/ml. As quinone fluorescence quenchers can also function as PS II electron acceptors, photoinhibition was routinely performed in the presence of DCMU (10 μM). The presence of DCMU during the photoinhibitory treatment did not influence the fluorescence parameters in control experiments. Fluorescence parameters were usually determined in a home-built fluorimeter with the emission wavelength near 685 nm, using 440 nm excitation, as previously described [12], and where specified in a commercial, pulse-modulated instrument (Heinz Waltz, Effeltrich, Germany). Measurements were performed after incubating phototreated samples in the dark for at least 10 min, sufficient to allow complete Q_A reoxidation, as judged by experiments with the oxidant potassium ferricyanide.

In Table 1A, the effects are shown of the photoinhibitory treatment of spinach thylakoids on F_0 (initial fluorescence), F_m (maximal fluorescence) and the F_v/F_m ratio (maximal quantum efficiency of stabile charge separation in PS II), where $F_v = F_m - F_0$. As is commonly observed [13], this leads to a marked decline of F_m, almost no change of F_0, and, as a consequence, a pronounced decrease in F_v/F_m. We have examined the influence of decreasing the excited state population in PS II, without modifying the incident light flux, on the photoinhibition-induced changes in fluorescence parameters. This was achieved by either inducing the 'spillover' transfer of energy from PS II to PS I by removing Mg^{2+} ions from the incubation medium [14–16] or by incubating thylakoids with 1 μM DBMIB, a quinone quencher of chlorophyll singlet excited states [17,18]. While in both cases the PS II excited state population was approximately halved under photoinhibitory conditions, as indicated by the F_m values (Table 1B,C), the percentage decrease in F_v/F_m due to photoinhibition was only slightly decreased. This situation, in which the relative decrease in F_v/F_m due to photoinhibition is only slightly sensitive to a 50% decrease in excited states, is clearly seen in Fig. 1 over a wide range of light fluences. This suggests that photoinhibition may be less sensitive to excited state levels than is usually thought.

As an important component of the F_v/F_m decline is associated with the large photoinduced quenching of F_m (Table 1), we have examined the sensitivity of this parameter to the excited state population. In Table 1 the effect of reducing the excited state population on F_m is shown. As indicated above, the lower fluorescence levels in the presence of DBMIB and in the absence of Mg^{2+} ions is due to the quenching processes induced by these treatments and indicates that the excited state population was approximately halved in both cases. The lower percentage of the photoinhibition-induced decrease in F_m in the presence of quinone and in the absence of Mg^{2+} ions (Table 1B,C) cannot be interpreted as a decrease in the photoinhibitory effect, as fluorescence measurements were performed in the presence of the quenching states designed to lower the excited state population (k_q and k_s in Eq. 1 below). Thus in order to quantify the photoinhibition-induced quenching, we have analysed F_m fluorescence in terms of Eq. 1:

$$F_m = \frac{k_f}{k_{tr} + k_i + k_s + Nk_q} \quad (1)$$

where k_f is the intrinsic rate constant for fluorescence; k_{tr} is the sum of all trivial, intramolecular excited state decay processes including fluorescence, thermal decay and intersystem crossing; k_i is the quenching rate process induced by photoinhibitory treatment; k_s is the spillover rate; k_q is the DBMIB-induced quenching rate as modulated by the quencher concentration, N. That this simple formalism yields an adequate description of F_m has already been demonstrated by several groups [18–20]. We have confirmed this under our conditions by finding a linear Stern–Volmer plot ($1/F_m$ versus

Table 1
Effect of reducing the excited state population on photoinhibition

	(A) 5 mM $MgCl_2$		(B) 0 M $MgCl_2$		(C) 5 mM $MgCl_2$+1 μM DBMIB		(D) 5 mM $MgCl_2$+4 μM DBMIB	
	Control	Photoinhibited	Control	Photoinhibited	Control	Photoinhibited	Control	Photoinhibited
F_0	23.6 ± 0.8	24.8 ± 1.4	24.0 ± 0.8	23 ± 2	18.5 ± 1.4	19 ± 2	22.9 ± 1.1	21 ± 2
F_M	100 ± 3	54 ± 4 (−46%)	54 ± 4	39 ± 3 (−27%)	48 ± 3	37 ± 2 (−23%)	95 ± 4	50 ± 3 (−48%)
F_V/F_M	0.77 ± 0.04	0.56 ± 0.03 (−27%)	0.55 ± 0.03	0.41 ± 0.02 (−25%)	0.62 ± 0.03	0.48 ± 0.03 (−23%)	0.76 ± 0.04	0.58 ± 0.03 (−22%)

The effect of reduction of the excited state population in PS II on the photoinhibition-induced changes in the fluorescence induction parameters in spinach thylakoids. Excited states in PS II were decreased by about 50% by either 'spillover' (column B) or DBMIB (columns C and D). The DBMIB concentration was 1 μM during both the photoinhibitory treatment and fluorescence measurement for column C, while in column D the photoinhibitory treatment was performed with 4 μM DBMIB which was subsequently diluted to 0.16 μM for the fluorescence determination. F_m and F_0 values are normalised to the control F_m of 100. The errors are given as standard deviations. The values in parentheses are the percentage decrease due to photoinhibition.

DBMIB concentration) over a tenfold range of F_m values (data not shown). For photoinhibition calculations the unquenched F_m yield (k_f/k_{tr}) in the presence of Mg^{2+} ions was taken as 0.1 with $k_f = 1$ in arbitrary calculation units. In this way k_s and k_q values of around 8.5 and 11 were determined when spillover and quinone quencher were present. The calculated k_i values due to the photoinhibitory treatment are shown in Fig. 2 as a function of photoinhibition time (Fig. 2A), light fluence (Fig. 2B) and excited state population (Fig. 2C). From Fig. 2A,B it can be seen that k_i is linear with both light fluence and photoinhibition time in the presence and absence of the quenching treatments, thus indicating that the so-called 'reciprocity' rule is obeyed. Of particular interest is that the photoinhibition-induced quenching parameter, k_i, is almost uninfluenced by halving the excited state population.

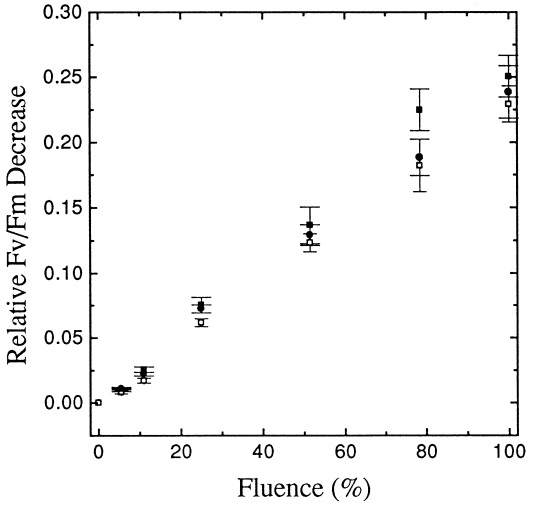

Fig. 1. The effect of decreasing the PS II excited state population on the photoinhibition-induced decrease in F_v/F_m as a function of the photoinhibitory light flux. Excited states were reduced by about 50% by spillover (0 mM $MgCl_2$; ●) or DBMIB (1 μM; □). Control thylakoids (■) were incubated with $MgCl_2$ (5 mM) present. Error bars are the standard deviations.

We have further analysed this by modulating the excited state population over a wide range by using different concentrations of DBMIB during the photoinhibitory treatment. The data in Fig. 2C indicate a small decrease in the calculated value for k_i upon reduction of excited states; however, the effect is only about 30% of that which would be expected on the basis of the 'reciprocity' rule.

The data analysis presented above strongly indicates that the rate process associated with F_m quenching (k_i) is only to a small extent dependent of the excited state population in PS II even though this parameter is linear with both light flux density and photoinhibition time. As this conclusion is rather surprising, we sought to confirm it by performing measurements in which the F_m changes in thylakoids with different excited state levels could be compared directly, i.e., without using the simple numerical analysis described above to determine k_i. To this end photoinhibitory treatments were performed at a high chlorophyll concentration (100 μg/ml). Under these conditions it was necessary to increase the DBMIB to 4 μM in order to halve the excited state population at F_m, as judged by measurements with the PAM fluorimeter. Subsequently samples were diluted 25 times and the fluorescence determined as usual. In this way the DBMIB concentra-

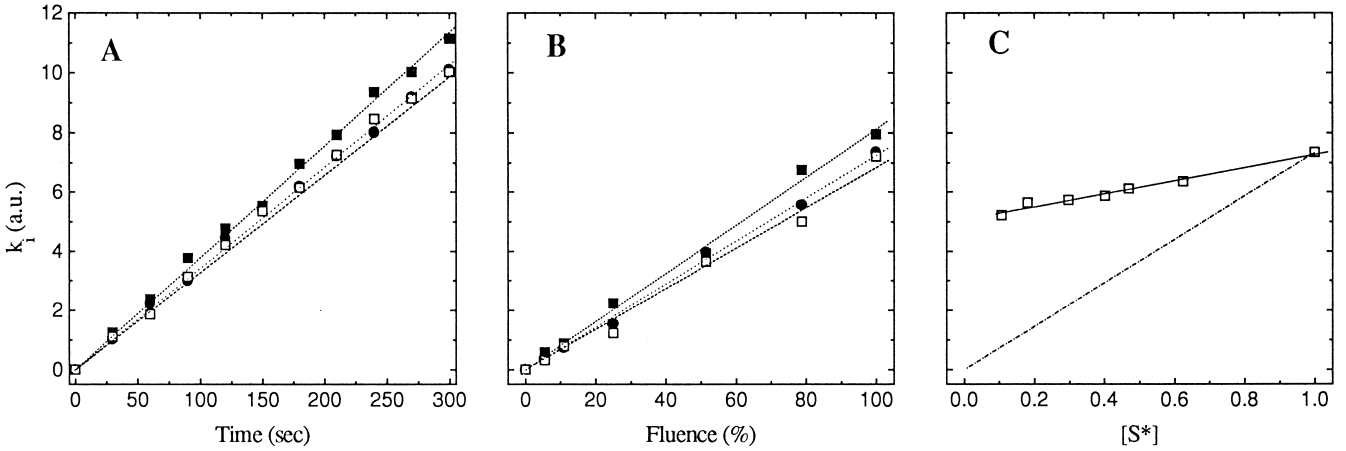

Fig. 2. Effect of decreasing the PS II excited state population on the photoinhibition-induced quenching rate process, k_i, by either DBMIB (1 μM) or spillover (0 mM $MgCl_2$). Data are presented for photoinhibition as a function of: (A) photoinhibition time, (B) light fluence and (C) relative excited state population as modified by DBMIB titration. In C, the dash-dotted line shows the predicted changes according to the 'reciprocity' rule. Symbols are as in Fig. 1. Linear fits are also shown.

tion present during fluorescence measurement was 0.16 μM and gave only a small quenching effect in itself (e.g., Table 1, compare control columns A and D). These data (Fig. 3; Table 1A,D) show that the photoinhibition-induced quenching of F_m was largely independent of the excited state population, thus directly confirming the conclusion stated above.

The substantial insensitivity of the photoinhibition-induced changes in fluorescence parameters to the singlet excited state population in PS II is difficult to understand, particularly as the underlying k_i process analysed above appears to be linear with respect to photoinhibition time and light flux density. In the following discussion we will briefly examine this point and suggest a possible explanation. Time-resolved transient absorption and fluorescence studies show that mainline energy transfer processes in chlorophyll binding antenna complexes occur on a picosecond and subpicosecond time scale (for review see [21]). Thus, thermal equilibration of excited states is extremely rapid and seems to occur within a few tens of picoseconds [21,22], i.e., about 10-times less than the mean excited state lifetime of PS II with open reaction centres and about 50-times less than for closed reaction centres. It is therefore expected that all antenna sites will be visited many times during the excited state lifetime. It has in fact been explicitly demonstrated that complete thermal equilibration of excited states occurs in PS II [23]. Thus it is evident that any antenna quenching process is expected to reduce the excited state population of the entire antenna system and should be equivalent to reduction of the absorption flux. The present observations that the fluorescence parameters associated with photoinhibition are modified according to the light 'reciprocity' rule but display a substantial insensitivity to the excited state population, as modified by quenching processes, are therefore not easy to reconcile. A possible way around this conundrum is

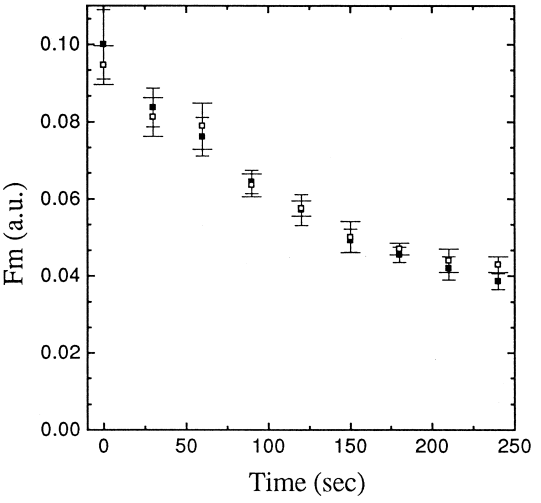

Fig. 3. Effect of decreasing the PS II excited state population on the photoinhibition-induced quenching of F_m fluorescence. Excited states were reduced by about 50% by incubation of chloroplasts (100 μg/ml chlorophyll) with DBMIB (4 μM; □). Control thylakoids (■) were incubated with $MgCl_2$ (5 mM) present. Error bars are the standard deviations.

to suggest that PS II contains a small population of uncoupled or poorly coupled chlorophylls. Such chlorophylls are substantially insensitive to quinone antenna quenchers [24] and spillover, though clearly their integrated photon absorption would be linear with both time and incident light fluence. In addition, as is the case for solvated chlorophyll, they would have a high triplet formation probability and these triplets would not be quenched by antenna carotenoids. Thus, as occurs for monomer, solvated chlorophyll, the probability of formation of activated oxygen species would be high. In the context of this suggestion it is interesting to note that Vasil'ev et al. [25] have recently suggested that the low-amplitude ($<1\%$), nanosecond component, commonly observed in PS II fluorescence decay experiments [25–27], may in fact be due to uncoupled chlorophylls. We are at present investigating this interesting possibility.

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